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Research Overview

P2Y Nucleotide Receptors in the Immune System: Signaling by a $P2Y_2$ Receptor in U937 Monocytes

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT G protein-coupled P2Y nucleotide receptors have been described in cells of the immune system, including neutrophils, monocytes, macrophages, B- and T-lymphocytes, granulocytes, and myeloblasts. In the monocyte/macrophage lineage, a $P2Y_2$ receptor subtype activated equipotently by adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) is coupled to phospholipase C and regulates low density lipoprotein uptake, superoxide production, gating of calcium channels, and phagocytosis. In U937 monocytes, $P2Y_2$ receptor activation leads to phosphorylation of MKK3 and p38, mitogen-activated protein kinases. $P2Y_2$ receptors in U937 monocytes undergo agonist-induced desensitization that decreases the potency and efficacy of subsequent doses of agonist. Cells recover rapidly from desensitization after short-term (<30 minutes) agonist treatments, whereas long-term (>1-hour) treatments produced sustained desensitization correlating with a decrease in $P2Y_2$ receptor mRNA levels. To investigate the molecular determinants of desensitization, a recombinant $P2Y_2$ receptor was expressed in human astrocytoma cells in which it exhibited agonist-induced desensitization and sequestration. $P2Y_2$ receptors containing C-terminal deletions of potential phosphorylation sites for protein kinases were resistant to desensitization and sequestration. Other results indicate that an integrin-binding domain, arginine-glycine-aspartate (RGD), in the first extracellular loop of the $P2Y_2$ receptor binds specifically to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (vitronectin receptors), an intriguing finding considering the wide distribution of these receptors among immune cells. The RGD domain was necessary for localizing the receptor to focal adhesion complexes to promote efficient receptor signaling. Finally, positively charged amino acids were identified in the ligand binding site of the $P2Y_2$ receptor, information that could promote the design of compounds for selective modulation of immune function. Drug Dev. Res. 45:222–228, 1998.

Key words: purinergic receptors; monocytes; UTP; $P2Y_2$ receptors; immune system

P2Y NUCLEOTIDE RECEPTORS IN THE IMMUNE SYSTEM

It is recognized that nucleotides including adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) mediate diverse biological responses in a variety of tissues by activation of specific cell surface P2 nucleotide receptors [Turner et al., 1998]. P2 nucleotide receptors have been characterized and classified by using pharmacologic and molecular approaches [Turner et al., 1998]. Two major families of P2 receptors have been identified: those belonging to the G protein-coupled receptor family termed P2Y, and those belonging to the ligand-gated ion channel family termed P2X [Fredholm et al., 1994].

G protein-coupled P2Y receptors have been characterized in B- and T-lymphocytes [Padeh et al., 1991; Koshiba et al., 1997], macrophages [Hagenlocker et al.,

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1990], monocytes [Cowen et al., 1989; Altieri et al., 1990], neutrophils [Kuhns et al., 1988; Kuroki et al., 1989], granulocytes [Cowen et al., 1991; Akbar et al., 1997], and myeloblasts [Clifford et al., 1997]. The cloning of several P2Y receptor subtypes [reviewed in Weisman et al., 1998] has facilitated the identification of P2Y receptors in the immune system. A $P2Y_1$ receptor subtype that is selective for adenine nucleotides is expressed in B-lymphocytes [Padeh et al., 1991], T-leukemia cells [Biffen and Alexander, 1994], and myeloblasts [Clifford et al., 1997]. A $P2Y_2$ receptor subtype that can be activated equipotently and equiefficaciously by ATP and UTP is expressed in macrophages [el-Moatassim and Dubyak, 1992, Murgia et al., 1993, Müller et al., 1993; Alonso-Torre and Trautmann, 1993; Murphy et al., 1993; Ichinose, 1995], monocytes [Cowen et al., 1991; Müller et al., 1993; Martin et al., 1997; Akbar et al., 1997], neutrophils [Martin et al., 1997; Gargett and Wiley, 1997], granulocytes [Cowen et al., 1991; Martin et al., 1997; Akbar et al., 1997], and activated T cells [Koshiba et al., 1997]. A uridine nucleotide-selective $P2Y_4$ receptor has been described in RAW 264.7 macrophages [Lin and Lee, 1996].

The monocyte/macrophage lineage plays an important role in immune function, and P2Y receptors regulate diverse functions in these cells. P2Y receptor activation causes phenotypic changes associated with differentiation of monocytes to macrophages [Cowen et al., 1991]. In macrophages, $P2Y_2$ receptors mediate decreased low density lipoprotein uptake [Müller et al., 1993], increased superoxide production [Murphy et al., 1993], the gating of calcium channels [Alonso-Torre and Trautmann, 1993; Naumov et al., 1995], and phagocytosis [Ichinose, 1995]. Monocyte/ macrophage P2Y receptor activation also stimulates cell adhesion [Ventura and Thomopoulus, 1995] and regulates the expression of inducible nitric oxide synthase in response to macrophage activators [Denlinger et al., 1996; Greenberg et al., 1997]. Recently, P2Y receptors have been reported to increase $\alpha_M\beta_2$ integrin expression in monocytes and granulocytes [Akbar et al., 1997].

The human promonocytic U937 cell line is a model progenitor cell line that has been extensively used for studies of myelomonocytic differentiation by a wide variety of physiologic and pharmacologic agents. For example, phorbol esters, vitamin D, and cytokines induce monocyte to macrophage patterns of maturation in U937 cells [Harris and Ralph, 1985]. Although the role for P2Y receptors in modulating myelomonocytic differentiation is appreciated [Cowen et al., 1991], the molecular/biochemical basis for these effects is yet to be identified and very little is known about the regulation and structure/function relationships of P2Y receptors in human monocytes and macrophages.

In macrophages, ATP also activates a $P2X_7$ receptor subtype (formerly known as P_{2Z}) that differs from other P2X receptors in that its activation can lead to the generation of plasma membrane pores [Steinberg et al., 1987], a phenomenon that we have well characterized [Weisman et al., 1984; Gonzalez et al., 1989a,b; Erb et al., 1990]. The $P2X_7$ receptor also mediates the activation of phospholipase D [el-Moatassim and Dubyak, 1993], the release of interleukin-1β [Ferrari et al., 1997] and has been proposed to induce macrophage fusion to form multinucleated giant cells [Chiozzi et al., 1997]. Activation of $P2X_7$ receptors has been postulated to cause apoptosis, or programmed cell death, but the relationship of this response to pore formation has been inadequately investigated. Leukocytes, monocytes, and macrophages are selectively removed from inflammatory tissue by apoptosis [Vermes and Haanen, 1994]. This process, therefore, is important for tissue healing and prevention of secondary damage and scarring after infection. The therapeutic regulation of apoptosis during and after inflammation offers new approaches for promoting rapid healing and reduction of unwanted pathologic by-products of inflammatory processes [Vermes and Haanen, 1994]. Elucidation of the molecular basis for P2 receptor function in the U937 monocyte/macrophage lineage will certainly provide novel strategies to regulate inflammation by means of pharmacotherapy.

$P2Y_2$ RECEPTOR IN U937 MONOCYTES

Our unpublished results with the U937 monocytic cell line indicate that phorbol esters or the bacterial endotoxin lipopolysaccharide (LPS) induce the macrophage phenotype, as previously described [Harris and Ralph, 1985] (Fig. 1). In both U937 monocytes and macrophages, addition of UTP or ATP causes a rapid IP_3-dependent increase in the concentration of intracellular free calcium ion, $[Ca^{2+}]_i$, typical for G protein-coupled receptors linked to phospholipase C. The EC_{50} for $P2Y_2$ receptor activation in U937 cells is 0.98 μM for UTP and 1.6 μM for ATP. Consistent with the role of a $P2Y_2$ receptor in this response, $P2Y_2$ receptor mRNA was detected in U937 monocytes and macrophages. Activation of the $P2Y_2$ receptor with UTP (100 μM) in U937 monocytes induced sequential phosphorylation of the mitogen-activated protein (MAP) kinases, MKK3 and p38 (Fig. 2), similar to the effect of LPS on these cells [Santiago-Peréz and González, unpublished results]. Phospholipase C activation by the G protein-coupled $P2Y_2$ receptor leads to the generation of diacylglycerol, an endogenous activator of protein kinase C (PKC), suggesting that PKC and/or a subunit(s) of G protein causes stimulation of the MAP kinase cascade. Although UTP, unlike LPS and PMA, does not cause morphologic differentiation, the activation of MAP kinases in U937 cells by UTP is noteworthy because inhibition of

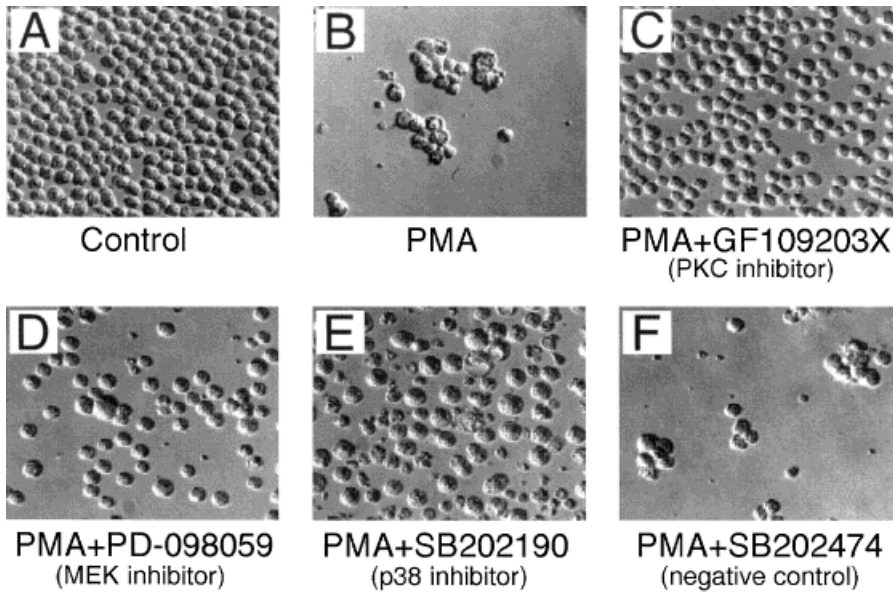

Fig. 1. Effect of protein kinase inhibitors on U937 cell differentiation. Morphologic differentiation of U937 cells was examined after a 72-hour incubation with (A) carrier, (B) 500 nM phorbol myristate acetate (PMA), (C) 500 nM PMA + 1 μM GF109203X (PKC inhibitor), (D) 500 nM PMA + 30 μM PD-098059 (MEK inhibitor), (E) 500 nM PMA + 10 μM SB202190 (p38 inhibitor), or (F) 500 nM PMA + 5 μM SB202474 (negative control for p38 inhibitor).

PKC, MEK, or p38 protein kinases blocked the PMA (500 nM)-induced differentiation of U937 cells (Fig. 1). These results also suggest that MAP-kinase activation is necessary but not sufficient for U937 monocyte differentiation.

P2Y receptors undergo agonist-induced desensitization [Wilkinson et al., 1994], similar to other members of the G protein-coupled receptor superfamily. P2Y receptor desensitization can be caused by treatment of cells with phorbol myristate acetate (PMA), an activator of protein kinase C (PKC) [Boarder et al., 1995]. For the G protein-coupled β_2-adrenergic receptor [Freedman and Lefkowitz, 1996], which is linked to adenylate cyclase, agonist-induced desensitization is mediated through receptor phosphorylation by G protein-coupled receptor kinases (GRKs). In U937 cells, $P2Y_2$ receptor-mediated calcium mobilization is rapidly desensitized by pretreatment with the agonist UTP (1–100 μM), or PMA (10–500 nM), as indicated by the decreased response to rechallenge with UTP (> 0.1 μM) within 5 minutes of the initial challenge (Table 1). It remains to be determined whether PKC, GRK, or both, mediate agonist-induced desensitization of $P2Y_2$ receptors in U937 cells, because potential phosphorylation sites for both kinases are present in the intracellular C-terminus of a recombinant $P2Y_2$ receptor (see below). Recovery from desensitization caused by UTP was found to be dependent on the concentration of the initial dose of UTP and the time between UTP additions [Flores and González, unpublished results]. Recovery from agonist-induced desensitization of G protein-coupled β_2-adrenergic receptors requires receptor internalization (sequestration) and dephosphorylation by intracellular phosphatases [Yu et al., 1993; Pippig et al., 1995], and it is likely that these pathways also participate in recovery of $P2Y_2$ receptor activity in U937 cells. Long-term desensitization of G protein-coupled receptors is associated with receptor down-regulation due to receptor degradation that

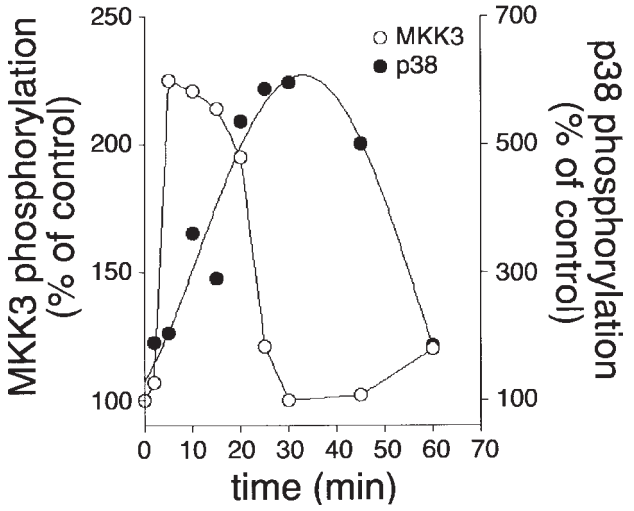

Fig. 2. MKK3 and p38 phosphorylation in U937 monocytes. The phosphorylation of MKK3 and p38 MAP kinases in response to 100 μM UTP at 37°C in U937 cells was detected at the indicated times by Western blot analysis by using specific antiphosphokinase (activated kinase) antibodies. The blots were stripped and re-probed with antikinase (total kinase) antibodies. The results are expressed as a percentage of the density obtained for total kinase.

TABLE 1. Response to Rechallenge with UTP[a]

	Response	
Pretreatment	EC_{50} value (μM)	Maximal response (%)
None	0.34	100
1 μM UTP	0.99	83
10 μM UTP	6.8	53
100 μM UTP	10.4	26
10 nM PMA	0.63	98
100 nM PMA	5.7	86
500 nM PMA	8.3	73

[a]U937 cells were pretreated with UTP or PMA at the indicated concentration for 5 minutes at 37°C. The cells were washed and retreated with various concentrations of UTP (0.01–500 μM) for 5 minutes at 37°C and the maximal increase in $[Ca^{2+}]_i$ was determined, as previously described [Garrad et al., 1998]. The effective concentration (EC_{50}) for induction of 50% of the maximal response was graphically determined. To obtain the maximal response, cells were rechallenged with 100 μM UTP after the indicated pretreatment and the peak response obtained was expressed as a percentage of the peak response to 100 μM UTP in control (no pretreatment) cells.

necessitates de novo protein synthesis to reestablish receptor levels. Consistent with these conclusions, U937 cells treated with millimolar concentrations of UTP exhibit persistent desensitization that is characteristic of receptor down-regulation. P2Y$_2$ receptor down-regulation in U937 cells is associated with a decrease in the level of P2Y$_2$ receptor mRNA [Santos-Berríos and Gonzáles, unpublished results].

STUDIES WITH A RECOMBINANT P2Y$_2$ RECEPTOR

Agonist-induced P2Y$_2$ receptor desensitization and resensitization in U937 cells likely involves receptor phosphorylation by protein kinases, sequestration from the plasma membrane, and resensitization by receptor dephosphorylation, whereas long-term desensitization is due to receptor down-regulation. It is difficult to study these pathways directly with endogenous P2Y$_2$ receptors in U937 cells because no receptor-selective antibodies are available to determine receptor protein levels in the plasma membrane or intracellular vesicles. In an attempt to learn more about the molecular mechanisms involved with P2Y$_2$ receptor desensitization, a recombinant P2Y$_2$ receptor [Lustig et al., 1993] was expressed in a clonal line of human 1321N1 astrocytoma cells [Erb et al., 1995; Garrad et al., 1998] at a level of ~20,000 molecules/cell [Garrad et al., 1998]. The 1321N1 cell line does not express functional P2Y receptors, making it a suitable model for these expression studies. The incorporation of a hemagglutinin (HA) epitope tag at the extracellularly oriented N-terminus of the P2Y$_2$ receptor enabled detection of the receptor by using an immunofluorescence assay and a commercially available anti-HA epitope antibody. Because the C-terminus of the P2Y$_2$ receptor contains two consensus phosphorylation sites for PKC (another site is present in the third intracellular loop) and potential phosphorylation sites for GRK, we investigated the role of this domain in agonist-induced receptor desensitization and sequestration by expressing a series of five truncation mutants of the recombinant receptor in 1321N1 cells. Results with the wild-type P2Y$_2$ receptor and truncated receptor mutants indicated that deletion of 18 or more amino acids from the C-terminus increased by approximately 30-fold (from 0.25 to ~8 μM) the minimal concentration of UTP necessary to desensitize the receptor [Garrad et al., 1998]. The rate and extent of UTP- (1 mM) induced P2Y$_2$ receptor sequestration and receptor resensitization also decreased as a function of truncation length [Garrad et al., 1998]. P2Y$_2$ receptor desensitization was maximal when >50% of the original receptor complement remained on the cell surface [Garrad et al., 1998]. Furthermore, receptor desensitization, but not sequestration, could be induced by PKC activation with PMA (1 μM). These results support the hypothesis that P2Y$_2$ receptor desensitization and sequestration occur by distinct mechanisms involving structural determinants within the C-terminal domain of the receptor.

Recent reports that P2Y receptor activation stimulates cell adhesion in macrophages [Ventura and Thomopoulus, 1995] and increases integrin expression in monocytes [Akbar et al., 1997] suggest that these receptors may have a previously unrecognized role in cell to cell communication. With respect to P2Y$_2$ receptors, the presence of the integrin-binding motif arginine-glycine-aspartic acid (RGD) in the first extracellular loop of recombinant P2Y$_2$ receptors [Lustig et al., 1993; Parr et al., 1994] could indicate that this P2Y receptor subtype can associate with integrins. Studies with an 18 amino acid peptide that matches the sequence of the RGD-containing domain of the P2Y$_2$ receptor indicate that this peptide when conjugated to fluorescent beads promotes bead binding to human K562 erythroleukemia cells that express α_5, α_v, β_1, β_3, and β_5 integrin subunits [Weisman et al., 1997; Erb et al., 1998]. This bead binding can be inhibited with the soluble RGD-containing peptide or with monoclonal antibodies to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (vitronectin receptors) but not with antibodies to the $\alpha_5\beta_1$ integrin (the fibronectin receptor). In comparison with results with the RGD-containing peptide, significantly less binding to K562 cells was obtained with beads conjugated with a similar peptide in which the RGD domain was replaced with RGE (arginine-glycine-glutamic acid), a motif that does not have high affinity for integrins [Pierschbacher and Ruoslahti, 1984]. These results suggest that the 18 amino acid RGD-containing peptide derived from the P2Y$_2$ receptor sequence is a ligand for vitronectin receptors expressed in K562 cells and that efficient peptide binding to the vitronectin receptor is dependent on the presence of the RGD domain.

The results with peptides encouraged attempts to determine whether the full-length $P2Y_2$ receptor has integrin-like activity in cells. Accordingly, we expressed in 1321N1 cells a mutant $P2Y_2$ receptor in which the RGD motif was replaced by RGE. Experiments with cell transfectants indicated that activation of the RGE-containing $P2Y_2$ receptor required a thousand-fold higher concentration of UTP or ATP (= 100 μM) than the wild-type RGD-containing $P2Y_2$ receptor [Erb et al., 1998]. These results suggest that association in focal adhesion complexes of the $P2Y_2$ and integrin receptors by means of the RGD domain may induce a high-affinity agonist-binding state of the $P2Y_2$ receptor to promote efficient signal transduction. Consistent with the hypothesis that $P2Y_2$ receptors can be components of focal adhesion complexes, it was determined that the wild-type $P2Y_2$ receptor colocalized with vinculin, a cytoskeletal protein exclusively found in focal adhesion complexes [Erb et al., 1998]. For these studies, the $P2Y_2$ receptor protein was detected in whole cells by using immunofluorescence assays with antibodies that recognize the HA epitope incorporated at the N-terminus of the receptor and, subsequently, vinculin was detected in fixed cells by using antivinculin antibody. In contrast to results with the wild-type $P2Y_2$ receptor, cells expressing the RGE-containing $P2Y_2$ receptor, or the turkey $P2Y_1$ receptor that lacks an RGD motif [Filtz et al., 1994] did not exhibit colocalization of receptors and vinculin. Taken together, these results indicate that association between the RGD-containing $P2Y_2$ receptor and focal adhesion complexes enables extracellular nucleotides to efficiently couple to intracellular signal transduction pathways. Because the $\alpha_v\beta_3$ vitronectin receptor is expressed in monocytes, macrophages, and neutrophils [Lindberg et al., 1996; Hendey et al., 1996; Hayashi et al., 1997; Hughes et al., 1997], there are potentially multiple interactions between $P2Y_2$ receptors and integrins that could occur in the immune system. Furthermore, the $\alpha_v\beta_3$ integrin receptor is expressed at high levels in angiogenic endothelial cells and antagonists of $\alpha_v\beta_3$ cause apoptosis and inhibition of angiogenesis [Brooks et al., 1994a,b], a process that is essential for embryonic development and wound repair. The $P2Y_2$ receptor is up-regulated in response to ligation of salivary gland [Turner et al., 1997] and as an immediate early gene response in activated thymocytes [Koshiba et al., 1997], and down-regulated during differentiation of human myeloid leukocytes [Martin et al., 1997]. Thus, relationships between $\alpha_v\beta_3$ and $P2Y_2$ receptor expression levels in vivo may have relevance to development and wound healing.

The 1321N1 cell expression system for $P2Y_2$ receptors has been useful for identifying amino acids in the ligand binding site of the receptor. By sequence comparison of the chick $P2Y_1$ receptor [Webb et al., 1993] and the murine and human $P2Y_2$ receptors [Lustig et al., 1993; Parr et al., 1994] positively charged amino acids were selected as potential ligand binding sites for the negatively charged phosphate groups of nucleotide agonists. When expressed in 1321N1 cells, three $P2Y_2$ receptor mutants with neutral amino acids replacing ^{262}His, ^{265}Arg, or ^{292}Arg exhibited diminished ATP and UTP potencies (EC_{50}s ~ 100, 450, and 850 μM for the respective receptor mutants, compared with 1 μM for the wild-type receptor) [Erb et al., 1995]. Charge conservation by substitution of ^{289}Lys with Arg in the $P2Y_2$ receptor increased the potencies of ADP (EC_{50} ~ 0.25 μM) and UDP (EC_{50} ~ 0.14 μM) and decreased potencies of ATP (EC_{50} ~ 300 μM) and UTP (EC_{50} ~ 26 μM), suggesting that minor sequence differences may underlie the differing agonist potency profiles exhibited by the various P2Y receptor subtypes. All of the P2Y receptors cloned to date have positively charged amino acids in the 6th and 7th transmembrane domains [Weisman et al., 1997], further evidence of the importance of this region to nucleotide binding. Recently, additional amino acids in the 3rd and 6th transmembrane domains have been identified that participate in ligand binding by the $P2Y_1$ receptor [Jiang et al., 1997]. By using data provided by mutagenesis studies, molecular models of the $P2Y_1$ and $P2Y_2$ receptors [Erb et al., 1995; van Rhee et al., 1998] have depicted the ligand binding site of P2Y receptors. This information will aid in the rational design of selective agonists and antagonists of P2Y receptors that could be used as effective agents for the treatment of immune disorders.

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